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Use of Synthetic Cardiolipin and Lecithin in the Antigen Used by the Venereal Disease Research Laboratory Test for Serodiagnosis of Syphilis

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The Venereal Disease Research Laboratory (VDRL) test is a microflocculation test for syphilis that uses an antigen containing cardiolipin, lecithin, and cholesterol. For more than 50 years, the preparation of natural cardiolipin and lecithin for this test has been based on the Pangborn method which involves isolating and purifying these components from beef hearts. This process is tedious and time-consuming and results in a variable purity range. In our studies, we found that a VDRL antigen using synthetic tetramyristoyl cardiolipin and synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (lecithin) was as specific in detecting syphilis as a VDRL antigen made with natural components. In 85% of the cases, we obtained an endpoint titer of 1/2 or 1 dilution more than a titer obtained with a VDRL antigen made with natural components. The use of these pure synthetic compounds, with a purity of 99%, would offer advantages in the standardization and stability of the VDRL antigen. Because this antigen is the basic ingredient in the preparation of nontreponemal reagents such as the rapid plasma reagin, toluidine red unheated serum test, and the unheated serum reagin, the use of this synthetic VDRL antigen should also increase the reactivity of these reagents.

The Venereal Disease Research Laboratory (VDRL) antigen, suspended in a buffered saline solution, forms a flocculate when combined with antilipoidal antibodies in serum or cerebrospinal fluid (CSF) from persons with syphilis. The VDRL test measures immunoglobulin G (IgG) and IgM antibodies to lipoidal material released from damaged host cells as well as to lipoprotein-like material and possibly cardiolipin released from the treponemes (1, 9).

Natural cardiolipin was isolated from extracts of beef hearts by Pangborn in 1941 (10). Natural lecithin is prepared from beef hearts or egg yolks. The process of preparing and purifying cardiolipin and lecithin by the Pangborn method has continued, despite being tedious, time-consuming, and very expensive.

Natural cardiolipin is a phospholipid containing approximately 90% unsaturated linoleoyl fatty acid with four 18-carbon chains, each of which has two double bonds. The presence of the double bonds in the fatty acid is conducive to oxidation with reduced reactivity. Cardiolipin possesses a strong antigenic property. The serologic specificity of the antigen reacting with syphilitic sera is attributed to two phosphate groups and the β-hydroxyl group of the central glycerol moiety (3). Several investigators have reported on the relationship between the structure and serologic specificity of cardiolipin. The length of the carbon chain of the fatty acids apparently is not of primary importance. Faure and Coulon-Morelec (4) demonstrated that the removal of one or two of the four fatty acids from the phosphatides derived from cardiolipin did not have a detrimental effect, while the esterification of the free hydroxyl group of cardiolipin considerably decreased the activity of the cardiolipin in the VDRL test.

Several researchers have sought to determine whether synthetic cardiolipin and lecithin could replace the natural components in the preparation of VDRL antigen (3, 5, 12, 13). These experiments were designed to demonstrate the possibility of obtaining the same serologic reactions both qualitatively and quantitatively with synthetic test antigen as with a reference antigen containing natural components. Their studies (3, 6, 13) showed that phosphatidylglycerophosphate, phosphatidylglycerol, phosphatidic acid, and diphosphatidylglycerol are less reactive than natural cardiolipin. In a separate study, several types of synthetic lecithin (L-α-distearoyl, dipalmitoyl, D-α-dimyristoyl, DL-α-dimyristoyl, and L-α-dimyristoyl cephalin) were produced and found to be serologically active to varying degrees (12). The results of these tests showed that the VDRL antigens prepared with these synthetic lecithins were significantly less sensitive than were those prepared with equivalent amounts of natural lecithin (12).

We conducted experiments to investigate the use of synthetic compounds in the preparation of VDRL antigen. The use of these pure synthetic compounds would offer advantages in the standardization as well as stability of the antigen.

MATERIALS AND METHODS

Preparation of synthetic VDRL antigen. We obtained an ethanolic solution of synthetic tetramyristoyl cardiolipin at a concentration of 6 mg/ml and another ethanolic solution of synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (lecithin) at a concentration of 40 mg/ml from Avanti Polar Lipids, Inc. (Alabaster, Ala.). We also obtained cholesterol with a purity of 98% from the same manufacturer.

The tetramyristoyl cardiolipin is synthesized from lipid precursors, purified by silica gel chromatography, and tested for purity by thin-layer chromatography and high-pressure liquid chromatography. The purity of the synthetic cardiolipin is greater than 99%. The synthetic lecithin product is synthesized from 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine by acylation of the second position with oleic acid. The purity of this product is greater than 99%. A synthetic VDRL

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antigen (patent pending) was made in an ethanolic solution containing 0.03% tetramyristoyl cardiolipin, 0.14% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, lecithin, and 0.9% cholesterol. Components were added in the following sequence: cardiolipin, lecithin, cholesterol, and ethanol to volume. Small amounts of ethanol were used to rinse down the flask after the addition of each component. The antigen was solubilized and stored at room temperature, for at least overnight, before testing. A current lot of the Centers for Disease Control and Prevention (CDC) reference VDRL antigen and a Becton Dickinson Microbiology Systems VDRL antigen (BDMS, Cockeysville, Md.), which were made from natural components, were used as a reference in the test procedures.

Serum samples. Frozen banked serum samples reactive for the nontreponemal tests as well as serum samples from documented cases of syphilis, diseases other than syphilis (DOS), and biological false positives (BFP), defined as nontreponemal test reactive and treponemal test nonreactive, were evaluated by the standard VDRL procedure (7) using both the synthetic and natural VDRL antigens. Reactive samples were confirmed by the SERODIA *Treponema pallidum* particle agglutination test (TP-PA) (Fujirebio America, Inc., Fairfield, N.J.). Nonreactive VDRL samples that were reactive with TP-PA were confirmed by the fluorescent treponemal antibody-absorption (FTA-ABS) test (5). All sera reactive in the qualitative VDRL test were also run in the quantitative test. We obtained 495 freshly drawn routine clinical specimens with no patient identifiers from the sexually transmitted disease and clinical laboratories of the Shelby Medical Center, in Alabaster, Ala.; the Jefferson County Health Department in Birmingham, Ala.; the Georgia Department of Human Resources in Atlanta; and the Federal University Hospital of Santa Catarina in Florianapolis, Brazil. These specimens were tested by qualitative and quantitative VDRL tests using the synthetic and natural VDRL antigens. Reactive specimens were confirmed by the TP-PA, FTA-ABS, or enzyme-linked immunosorbent assay for IgG antibody (Wampole Labs, Cranbury, N.J.).

VDRL slide flocculation test. Freshly prepared emulsions of the synthetic and natural VDRL antigens were prepared daily according to the standard VDRL procedure (7). Serum samples were heat inactivated for 30 min at 56°C. In the qualitative test, 50 μl of each serum sample was placed into corresponding paraffin- or ceramic-ringed slides and a drop (17 μl) of each of the antigens was placed in the corresponding ring of the slide. The slides were placed in a mechanical rotator, rotated for 4 min at 180 rpm, and then read microscopically. The degree of flocculation of the two antigens was observed and recorded. In the quantitative test, serum samples were diluted twofold in a test tube with 0.9% saline. Fifty microliters of each of the tube dilutions was transferred to the corresponding rings of a ceramic- or paraffin-ringed slide, and a drop (17 μL) of each of the antigens was placed in the corresponding ring of the slide. The slides were again placed in a mechanical rotator and rotated for 4 min at 180 rpm. The endpoint titer of each of the serum dilutions was read microscopically. One doubling dilution difference was defined as an endpoint either reactive or weakly reactive for one antigen and weakly reactive or negative for the other antigen. One-half-dilution difference was defined as the difference among reactive strong, reactive, or reactive minus and the difference among weak strong, weak, or weak minus.

RESULTS

We found that synthetic tetramyristoyl cardiolipin, which contains four carbon chains of saturated fatty acids but no oxidation sites to reduce its reactivity, can be used as a substitute for natural cardiolipin in the preparation of VDRL antigen.

Of the several types of synthetic lecithin tested, we found that the asymmetrical fatty acid lecithin 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, containing one saturated 16-carbon fatty acid and one unsaturated 18-carbon chain with one double bond, could be substituted for natural lecithin.

The CDC VDRL antigen containing the synthetic cardiolipin and the synthetic lecithin was demonstrated to be more reactive than a VDRL antigen made with natural components. In the qualitative test, 100 frozen banked sera reactive by the nontreponemal (RPR) test were tested for comparison using the CDC synthetic VDRL antigen and CDC reference VDRL antigen. All (100%) were reactive with CDC synthetic VDRL antigen, and 88% were reactive with the CDC reference VDRL antigen. One sample out of 100 was found nonreactive with the confirmatory TP-PA but reactive with both VDRL antigens (Table 1).

The 100 serum samples from documented syphilis cases were used to compare the CDC synthetic VDRL antigen and the BDMS reference VDRL antigen (Table 1). Of the nine samples from persons with untreated primary syphilis, one was nonreactive with TP-PA and FTA-ABS but reactive with both VDRL antigens. One of the six latent untreated samples was nonreactive with both antigens. Of the 15 samples from persons with treated primary syphilis, 12 were reactive and 3 were nonreactive with the CDC synthetic VDRL antigen and 11 were reactive and 4 were nonreactive with the BDMS VDRL antigen. Of the 20 samples from latent treated cases, 3 were nonreactive with the BDMS VDRL antigen and 2 were nonreactive with the CDC synthetic VDRL antigen. However, one of these samples that was reactive for both VDRL antigens was nonreactive with TP-PA.

TABLE 1. Comparison of frozen banked serum specimens from persons with documented and undocumented cases of syphilis[a]

Syphilis category	No. of serum samples			
	Total	Reactive with:		
		Synthetic VDRL antigen	Natural VDRL antigen[b]	TP-PA
Undocumented	100	100[c]	88[c]	99
Documented				
Untreated				
Primary	9	9	9	8
Secondary	20	20	20	20
Latent	6	5	5	6
Treated				
Primary	15	12	11	13
Secondary	30	30	30	30
Latent	20	18	17	19
Total	200	194	180	195

[a] All specimens were tested with CDC synthetic VDRL antigen. Undocumented sera were tested using the CDC reference VDRL antigen; documented sera were tested with BDMS VDRL antigen.

[b] Natural VDRL antigen was either CDC reference VDRL (undocumented serum samples) or BDMS VDRL antigen (documented sera).

[c] One sample was reactive with both VDRL antigens but nonreactive with TP-PA.

In the quantitative testing, 85% of the frozen banked sera reactive in the RPR test had endpoint titers of 1/2 or 1 dilution greater with CDC synthetic VDRL antigen than with the CDC reference VDRL antigen. In 15% of the cases, the endpoint titer obtained with the CDC synthetic VDRL antigen was equal to that obtained with the CDC reference antigen. In no sample tested was the endpoint titer greater with the CDC reference antigen than with the CDC synthetic VDRL antigen (Table 2).

In the quantitative test, the endpoint titer obtained with the CDC synthetic VDRL antigen was found to be 1/2 or 1 dilution greater than that obtained with BDMS VDRL antigen in 84% of the documented cases of syphilis. In 7% of the cases, the endpoint titer of the two antigens was equal, while in 3% of the cases the endpoint titer of the BDMS reference VDRL antigen was 1/2 or 1 dilution greater than that of the CDC synthetic VDRL antigen. All VDRL results on clinical specimens were confirmed by the TP-PA test (Table 2).

When the 100 clinical specimens from patients with DOS were tested qualitatively with CDC synthetic VDRL antigen and the BDMS VDRL antigen, all samples were nonreactive with both antigens. All of the specimens were also tested by TP-PA. Four were reactive in the TP-PA but nonreactive in the FTA-ABS test (Table 3).

The results of the CDC synthetic and the BDMS VDRL antigen testing of serum samples from the 50 individuals originally classified as BFP (nontreponemal test reactive and

TABLE 2. Comparison of titers 1/2 or 1 dilution greater in endpoint of frozen banked sera from undocumented and documented cases of syphilis, tested with CDC synthetic VDRL antigen and with CDC and BDMS reference VDRL antigens

Syphilis category	No. of specimens			
	Total	With CDC synthetic VDRL antigen higher	With natural VDRL[a] antigen higher	With CDC synthetic and natural VDRL antigen endpoints equal
Undocumented	100	85	0	15
Documented				
Untreated				
Primary	9[b]	3	2	4
Secondary	20	20	0	0
Latent	6[c]	4	0	1
Treated				
Primary	15[d]	10	0	2
Secondary	30	30	0	0
Latent	20[e]	17	1	0
Total	200	169	3	7

[a] Natural VDRL antigen was either CDC reference VDRL (undocumented serum samples) or BDMS VDRL antigen (documented sera).
[b] One sample was nonreactive with TP-PA but reactive with both VDRL antigens.
[c] One sample was reactive with TP-PA but nonreactive with both VDRL antigens.
[d] One sample was reactive with TP-PA but nonreactive with both VDRL antigens, and two samples were nonreactive with TP-PA and both VDRL antigens.
[e] One sample was reactive with TP-PA but nonreactive with both VDRL antigens, and one sample was nonreactive with TP-PA and both VDRL antigens.

treponemal test nonreactive) are given in Table 4. Four serum samples that were originally misclassified as BFP were found reactive with the CDC synthetic VDRL antigen, the TP-PA, and the FTA-ABS. Three of these four samples were also reactive with the BDMS VDRL antigen.

We tested 495 clinical specimens with no patient identifiers with CDC synthetic VDRL antigen and the BDMS VDRL antigen and used the TP-PA, the enzyme-linked immunosorbent assay for syphilis IgG antibody, or the FTA-ABS as confirmatory tests on reactive specimens. For the 38 serum samples that were reactive in one of the treponemal tests, all were reactive with the CDC synthetic VDRL antigen and 36 were reactive with the BDMS VDRL antigen. Of the 457 serum samples nonreactive in the treponemal test, 452 were nonreactive with the CDC synthetic VDRL antigen and 450 were nonreactive with BDMS VDRL antigen (Table 5).

TABLE 4. Reactivity of 50 serum samples from cases of documented BFP tested qualitatively with CDC synthetic and BDMS VDRL antigen

Reactivity of sample	No. of samples with result for antigen:		
	TP-PA	CDC synthetic VDRL antigen	BDMS VDRL antigen
Reactive	4[a]	28	27
Nonreactive	46	22	23

[a] Four of these samples were reactive in the TP-PA and FTA-ABS and with the CDC synthetic VDRL antigen. Three of these four sera were reactive with BDMS VDRL antigen, and one was nonreactive.

DISCUSSION

For more than 50 years, the isolation and purification of cardiolipin and lecithin derived from beef hearts have been the method of choice for the preparation of natural VDRL antigen for the diagnosis of syphilis. Although the development of reliable synthetic substitutes has been the objective of several studies, previous results have indicated that VDRL antigen prepared with synthetic components is less reactive than that made with natural components (3, 12, 13). We found that a VDRL antigen made with synthetic tetramyristoyl cardiolipin and synthetic lecithin (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was more reactive than and as specific as the VDRL antigen made from natural components. The purity of the synthetic cardiolipin and lecithin is greater than 99% versus a variable purity range of 52 to 79% for cardiolipin, 80 to 91% for egg lecithin, and 60 to 86% for beef heart lecithin derived by the Pangborn method (11). This increased purity allows for an increase in reactivity without the usual corresponding increase in nonspecificity and gives a more stable product. Much of the instability of the natural components is due to oxidation of both the impurities and the double bonds in the carbon chains. Natural cardiolipin is a phospholipid containing approximately 90% unsaturated linoleoyl fatty acid with four 18-carbon chains, each of which has two double bonds, while synthetic tetramyristoyl cardiolipin has four saturated 14-carbon chains with no double bonds.

The synthetic VDRL antigen also tended to be more reac-

TABLE 3. Reactivity of CDC synthetic VDRL antigen and BDMS VDRL antigen with serum from patients with DOS and with no known history of syphilis

Category of Sample	No. of specimens	No. of serum samples reactive (R) and nonreactive (N) with:							
		CDC synthetic VDRL antigen		BDMS VDRL antigen		TP-PA		FTA-ABS	
		R	N	R	N	R	N	R	N
Rheumatic fever	27	0	27	0	27	4	23	0	27
Coronary arterial disease	9	0	9	0	9	0	9	ND[a]	ND
Hypertension	6	0	6	0	6	0	6	ND	ND
Diabetes	4	0	4	0	4	0	4	ND	ND
Parkinson's disease	2	0	2	0	2	0	2	ND	ND
Obesity	2	0	2	0	2	0	2	ND	ND
Angina	2	0	2	0	2	0	2	ND	ND
Miscellaneous category	48	0	48	0	48	0	48	ND	ND
Total no.	100	0	100	0	100	4	96		

[a] ND, not determined.

TABLE 5. Reactivity of 495 clinical specimens from sexually transmitted disease clinics that were tested qualitatively with CDC synthetic VDRL antigen and BDMS VDRL antigen[a]

Test and result	No. of Tests	No. of samples with result for antigen:			
		CDC synthetic VDRL antigen		BDMS VDRL antigen	
		Reactive	Nonreactive	Reactive	Nonreactive
Treponemal; reactive	38	38	0	36	2
Nontreponemal; nonreactive	457	5	452	7[b]	450

[a] A treponemal test was run as the confirmatory test on reactive specimens.
[b] Five of these seven sera were also reactive with the CDC synthetic VDRL antigen.

tive than the antigen made with natural components, with titers being from 1/2 to 1 dilution higher in 85% of the frozen banked serum samples tested and 84% of the documented cases of primary, secondary, or latent syphilis.

In monitoring the efficacy of treatment, it is recommended that serum samples be run with the same test and in the same laboratory in which the first serum sample was tested (1). When using the synthetic VDRL antigen, it is necessary to use the same reagent to test the initial and follow-up samples. A fourfold drop in titer using natural VDRL antigen is considered evidence of adequate treatment. In late latent syphilis, nontreponemal antibodies may disappear even without treatment.

We tested 50 serum samples that originally had been classified as BFP in the nontreponemal tests. Four of these samples were reactive in the TP-PA; while these may be false positives, it is more likely that they were misclassified when originally documented. Results from qualitative VDRL testing on these four serum samples ranged from reactive to weak with the synthetic VDRL antigen and from reactive to nonreactive with the reference antigen.

Studies are presently under way to test the synthetic VDRL antigen in the VDRL-CSF test, the unheated serum reagin, the RPR, and the toluidine red unheated serum test. The VDRL-CSF test is the only test approved for testing spinal fluid for the diagnosis of neurosyphilis. The currently used natural antigen is only about 50% sensitive in the VDRL-CSF test, with a range of 10% for asymptomatic cases to 90% for symptomatic cases (8). Any increase in sensitivity would be an advantage since neurosyphilis is difficult to diagnose. The VDRL-CSF test is highly specific, so that a reactive test is diagnostic for neurosyphilis. The RPR is currently the most used nontreponemal test in the United States and one of the most sensitive nontreponemal tests. Any increase in reactivity would offer the chance to detect more cases of untreated syphilis.

Our VDRL antigen made with synthetic cardiolipin and synthetic lecithin had a sensitivity similar to that of the commercial VDRL antigen on testing of 645 samples including those from persons with DOS, BFP, and routine samples from clinical laboratories. Compared to the commercial VDRL antigen, the synthetic antigen had a higher level of reactivity with 85% of the frozen banked samples tested and 84% with samples from documented cases of syphilis. Because VDRL antigen is the basic ingredient in the preparation of unheated serum reagin, RPR, and toluidine red unheated serum test antigens, the increase in reactivity of the synthetic VDRL antigen may also serve to improve the usefulness of these reagents in the detection of of nontreponemal antibodies in persons with syphilis.

REFERENCES

1. **Belisle, J. T., and M. E. Brandt.** 1994. Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. J. Bacteriol. **176:**2151–2157.
2. **Centers for Disease Control and Prevention.** 1998. Guidelines for treatment of sexually transmitted diseases. Morb. Mortal. Wkly. Rep. **47**(no. RR-1)**:** 28–47.
3. **De Haas, G. H., and L. L. M. Van Deenen.** 1965. Chemical structure and serological activity of synthetic and natural cardiolipin. Nature **206:**935.
4. **Faure, M., and M. J. Coulon-Morelec.** 1963. Rapports entre la structure chimique du cardiolipide et son activite serologique. Ann. Inst. Pasteur. **104:**246–263.
5. **George, R. W., E. F. Hunter, and M. B. Fears.** 1998. Fluorescent treponemal antibody-absorption (FTA-ABS) test, p. 225–245. *In* S. A. Larsen, V. Pope, R. E. Johnson, and E. J. Kennedy, Jr. (ed.), A manual of tests for syphilis, 9th ed. American Public Health Association, Washington, D.C.
6. **Inoue, K., and S. Nojima.** 1967. Immunochemical studies of phospholipids. Chem. Phys. Lipids. **1:**360–367.
7. **Kennedy, E. J., Jr., and E. T. Creigton.** 1998. Venereal disease research laboratory (VDRL) slide test, p. 157–178. *In* S. A. Larsen, V. Pope, R. E. Johnson, and E. J. Kennedy, Jr. (ed.), A manual of tests for syphilis, 9th ed. American Public Health Association, Washington, D.C.
8. **Larsen, S. A., E. A. Hambie, G. H. Wobig, and E. J. Kennedy.** 1985. Cerebrospinal fluid serologic test for syphilis: treponemal and nontreponemal tests, p. 157–162. *In* R. Morisset and E. Kurstak (ed.), Advances in sexually transmitted diseases. VNU Science Press, Utrecht, The Netherlands.
9. **Matthews, H. M., T. K. Yang, and H. M. Jenkin.** 1979. Unique lipid composition of *Treponema pallidum* (Nichols virulent strain). Infect. Immun. **24:**713–719.
10. **Pangborn, M. C.** 1941. A new serologically active phospholipid from beef heart. Proc. Soc. Exp. Biol. Med. **48:**484.
11. **Reeves, M. W., B. E. McGrew, B. McLaurin, and L. Pine.** 1981. Relationship of phospholipid chemistry to serological reactivity in the Venereal Disease Research Laboratory slide test antigen. J. Clin. Microbiol. **14:**48–54.
12. **Reyn, A.** 1956. Use of synthetic, crystalline, dimyristoyl lecithin in cardiolipin antigens. Bull. W. H. O. **14:**567–576.
13. **Tonks, D. B., and R. H. Allen.** 1953. The use of some synthetic phosphatides in antigens for the serodiagnosis of syphilis. Science **118:**55–56.